

Protocol for the Evaluation of Bactericidal Activity of Antimicrobial Coated Surfaces

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I. Overview

This document describes a testing protocol to support the registration of antimicrobial surface coating products with non-food contact sanitizer claims. The following items summarize the approach employed to support these product claims:

1. This protocol is designed to address sanitizer claims for non-food contact coated surface products including a claim for “continuous reduction” of bacteria
2. The protocol is applicable to surface paint/coating products that are intended for indoor use only
3. All testing should be conducted under Good Laboratory Practice Standards
4. Efficacy testing involves the evaluation of two product production lots against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the treated test carriers from one of these lots having undergone the simulated wear process. The untreated control coupons do not receive the simulated wear procedure.
5. An effective product is expected to achieve a 3 log₁₀ reduction (LR) in viable bacteria between treated test carriers and untreated control carriers for each microbe within a 2 hr contact period
6. The protocol has been established for use with surface coating products, however, upon consultation with the EPA, the protocol may also be appropriate for testing other solid, non-food contact surfaces for antimicrobial activity
7. Product performance testing should be conducted on two production lots; one lot with sets of exposed carriers and the second lot with only unexposed carriers. The term “exposed” refers to carriers subjected to the simulated wear procedure, while the “unexposed” carriers refers to those not subjected to the wear procedure. The term “treated carriers” refers to coated carriers that contain the active ingredient (Corning® Guardiant® Antimicrobial Particles), while the term “untreated carriers” refers to coated carriers that do not contain the active ingredient. The second production lot will also test 10 additional paints that contain colorants 1-10 added to base paint. Colorant-containing samples will not be subjected to simulated wear procedure.
8. Paint with eggshell sheen will be tested by this protocol. Additional sheens will be tested with one lot of unexposed treated test carriers and unexposed untreated control carriers prepared from base paint. Performance criterion will be a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of each test microbe
9. Additional optional organisms (Appendix A) will be tested with one lot of unexposed treated test carriers and unexposed untreated control carriers prepared from base paint. Performance criterion will be a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of each test microbe. For microbes

that recover only 3 logs on control coupons, performance criterion will be a $\geq 99\%$ reduction with complete reduction of bacteria down to limits of detection.

Table 1 provides an overview of carrier testing requirements.

Table 1. Carrier distribution of coated surface products

Product Lot	Paint	Test Carriers per Microbe	Control Carriers per Test Microbe
Lot I	Base paint	5 exposed, treated test carriers	3 unexposed, treated test carriers; 3 unexposed, untreated control carriers
Lot II	Base Paint	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 1	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 2	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 3	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 4	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 5	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 6	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 7	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 8	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 9	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
	Base Paint, Colorant 10	5 unexposed, treated test carriers	3 unexposed, untreated control carriers
Viability (reference) controls	Uncoated scrub chart		3 carriers

II. Test Methodology

A. Carriers

1. For testing of two microbes from one production lot, prepare 10 exposed, treated test carriers, 6 unexposed, treated test carriers and 6 unexposed, untreated control carriers; and from a second production lot, prepare 10 unexposed, treated test carriers and 6 unexposed, untreated control carriers. 10 unexposed, treated test carriers each containing base paint with colorants 1-10 will also be prepared along with 6 corresponding unexposed, untreated control carriers. Extra carriers should be prepared for sterility assessment. The composition of the treated test carriers must be representative of the final product and meet the specifications for the target chemistry formulation. The chemical composition of the treated test product carriers must be documented.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Each carrier will be physically screened to insure uniformity. Carriers with visible surface or edge abnormalities (chipping, gouges, pits or deep striations etc.) should be discarded. The screening will be conducted prior to the wear cycles
5. Uncoated scrub chart panels are used as an organism viability control
6. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up
7. To monitor the occurrence of microbial contamination, randomly select an untreated control and treated test carrier from each batch and incubate in appropriate growth medium as a sterility control. No growth is the desired outcome
8. Provide details of physical screening and sterility check in the final report; coupon thickness and sheen will also be reported
9. Use decontaminated carriers within one week of preparation
10. All coupons are single use
11. Production lot (batch) identity will be maintained throughout the testing process

B. Simulated wear procedure

The simulated wear procedure is intended to represent a degree of normal and relevant physical wear, as well as reproduce potential effects resulting from repeated exposure of Guardiant® particle-containing surfaces to cleaning materials over a period of 4 years.

The wear exposure will be performed with a Gardco, Model D10V abrasion tester. The weight of the fully assembled abrasion boat (Gardco WA-2225) is to be kept between 1000g and 1085g

Simulated wear cycling will be initiated after the final coat of paint on each treated test panel has been allowed to cure for at least 24 hours. The wear procedure is intended to simulate cleaning of a vertical surface; therefore, relatively low volumes of cleaner are applied to the test panels at a rate where the cleaner would not drip down to the floor if applied to a vertical surface.

A cleaning solution is prepared using Best Yet™ citrus cleaner (or similar multipurpose) detergent at the manufacturer's recommended dilution ratio. A common sponge is soaked in the prepared cleaner solution prior to the initial simulated wear cycle. The appropriate test or control panel is placed on the Gardco D10V abrasion tester. The sponge is removed from the solution, wrung of excess solution and is positioned under the fully assembled abrasion boat. The sponge/abrasion boat combination is then attached to the Gardco tester on top of the secured panel. The abrasion tester speed is set between 2.25 to 2.5 for a total surface contact time of approximately 4 seconds per treatment.

The cycle is started, and two cycles are performed, representing one complete wear cycle. One complete wear cycle is equivalent to 4 washes or one month of cleaning. After the wear cycle is completed, weight boat and sponge are removed, and the sponge is placed in the cleaner solution. The panel is removed from the Gardco tester and placed on a horizontal surface to dry at room temperature. The panel is dried for ≥ 10 minutes before the next wear cycle is performed. It is visually verified to be dry before starting the next wear cycle. This procedure is repeated until a total of 48 wear cycles (total of $48 \times 4 = 192$ washes) have been performed on each panel. After the completion of 48 wear cycles, carriers are cut into 1" x 1" coupons as previously described. Product performance testing should be initiated within 3 days. All carrier storage conditions (temperature and humidity range) should be included in the study report.

C. Microbe Cultures

The test microbes are *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442)

1. **Stock cultures:** Initiate new stock cultures from lyophilized cultures from ATCC at least once every 18 months. Open ampule of freeze-dried organism per manufacturer's instructions
 - a. Using a tube containing 5-6 mL of tryptic soy broth (TSB), aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at $36 \pm 1^\circ\text{C}$ for 24 ± 2 hours
 - b. After incubation, streak a loopful of the suspension on tryptic soy agar (TSA) to obtain isolated colonies. Incubate the plates for 18-24 h at $36 \pm 1^\circ\text{C}$
 - c. Select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa* – the stock culture should be representative of all phenotypes present on the streak isolation plate. Spread plate 0.1 mL of the suspension on each of the 6-10 TSA plates. Incubate the plates for 18-24 h at $36 \pm 1^\circ\text{C}$
 - d. Following the incubation of the agar plates, place approximately 5 mL sterile cryoprotectant solution on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 tube may be used is necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture. Immediately after mixing, dispense 0.5-1 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures
 - e. Store the cryovials at $-70 \pm 5^\circ\text{C}$ for a maximum 18 months then reinitiate with a new lyophilized culture
 - f. Conduct Quality Control check of the pooled culture concurrently with freezing. For examples, streak a loopful on a blood agar plate, and selective media such as mannitol salt agar (MSA) and Cetrimide. Incubate all plates at $36 \pm 1^\circ\text{C}$ for 24 ± 2 hours. Record the colony morphology as observed on the blood agar plates and selective media plates (including the absence of growth). Perform a Gram stain from growth taken from the blood agar plates and observe the Gram reaction by using bright field microscopy at 1000x magnification (oil immersion)

2. Test Cultures

- a. For *S. aureus*, defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB

and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use

- b. For *P. aeruginosa*, defrost a single cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10mL TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hr. Inspect culture prior to use. Remove visible pellicle on surface of medium and around associated interior surfaces of the tube by pipetting or with vacuum suction. Using a serological pipette, withdraw the remaining broth culture (approx. 7-8 mL), avoiding any sediment on the bottom of the tube, and transfer to a new tube. Following removal of pellicle, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use
- c. Dilute in Phosphate Buffered Saline (PBS) or concentrate the culture appropriately to achieve the target carrier counts (4-5 logs/carrier). Centrifuge the 18-24 h broth cultures to achieve the desired level of viable cells on the dried carrier. Centrifuge at $\sim 5000 \text{ gN}$ for $20 \pm 5 \text{ min}$ and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet. For *S. aureus*, disrupt the pellet using vortexing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in 10 mL. If necessary, add 1mL of PBS to the pellet to aid in disaggregation
- d. Purity of the final test culture (with soil load) should be determined by streak isolation on TSA with 5% sheep's blood, or other appropriate plating medium, incubate ($36 \pm 1^\circ\text{C}$ for $48 \pm 4 \text{ hr}$), examine for purity
- e. Titer of the final test culture (with soil load) will be determined for informational purposes. Plate dilutions on TSA plates or other appropriate medium and incubate ($36 \pm 1^\circ\text{C}$ for 24-48 hr) and enumerate. Count the number of colonies to determine the number of organisms per mL (i.e. CFU/mL) of the inoculum present at the start of the test

D. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

E. Efficacy Test Procedure

1. Evaluate 5 exposed, treated test carriers (from one production lot) with 3 unexposed, treated test carriers and 3 unexposed, untreated control carriers against each test organism; and from the second production lot test 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers against each test organism
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μL of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

F. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

G. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

H. Product Performance Data

Impact of simulated wear

Production Lot 1

1. Comparative visual observations should be used to identify any deleterious effects caused by simulated wear for production lot 1; report findings in the study report
2. The effects of the simulated wear on mean log reduction should be presented for production lot 1 – this is based on the mean log reduction values for the exposed, treated test carriers compared to the mean log reduction values for unexposed, treated test carriers. The mean control counts associated with the unexposed, untreated control carriers are used for log reduction calculations
3. The mean log reduction values (i.e. per abrasion/chemical treatment per microbe) for the exposed, treated test carriers compared to the unexposed, treated test carriers should be within 0.5 log; in addition, the mean log reduction for the exposed product carriers should not be less than the performance standard of 3 logs for simulated wear group for either of the test microbes

Production Lot 2

Mean log reduction data for production lot 2 should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered a sanitizer, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of each test microbe (the difference between treated test carriers and the untreated control carriers)

I. Calculations/Data Analysis

Calculate the mean log reduction in viable cells for each microbe for the following treatments:

1. Exposed, treated test carriers for production lot 1
2. Unexposed, treated test carriers (one 3-carrier set per microbe) for production lot 1, and
3. Unexposed, treated test carriers for production lot 2

Log reduction values are calculated based on the difference in log densities associated with the exposed/unexposed treated test carriers compared to the unexposed, untreated control carriers.

1. For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

2. For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

3. For determining the geometric mean of number of organisms surviving on five treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

The equation will be modified for three unexposed treated test carriers

4. Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

5. Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on exposed/unexposed treated test carriers)

6. Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results)

Used for neutralization confirmation control only

J. Optional Label claims

The following label claims are supported by this protocol. Claims are limited to indoor use of hard, coated surface products.

1. This surface kills at least 99.9% of bacteria after a 2-hour contact time when maintained in accordance with the product care and use directions
2. Painted surfaces kill greater than 99.9% of Gram-negative and Gram-positive bacteria [within] [after] 2 hours of exposure*
3. Kills [pathogenic] [disease causing] [harmful] bacteria on [painted surfaces] *
4. Painted surfaces kill disease causing bacteria [within] [after] 2 hours of exposure
5. EPA-registered paint that continuously kills 99.9% of *Staphylococcus aureus* and *Pseudomonas aeruginosa*
6. Helps prevent the spread of bacteria on painted surfaces
7. Kills at least 99.9% bacteria* after a 2-hour contact time when maintained in accordance with the product care and use directions
8. [Antimicrobial][Microbicidal] paint
9. Painted surfaces continuously reduce bacterial contamination, achieving 99.9% reduction of bacteria [within] [after] 2 hours of exposure, and continue to kill 90% of bacteria even after repeated contamination
10. Microbicidal efficacy lasts for four years as long as the integrity of the surface is maintained
11. Kills ESKAPE pathogens[†]
12. Kills Staph, E. coli, Salmonella, Strep[‡]
13. Kills antibiotic-resistant bacteria on painted surfaces**

**Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*

[†]Acronym for *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella Pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, bacteria that can cause infections

[‡]*Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, *Streptococcus pyogenes*

***Carbapenem-resistant Enterobacter cloacae*, *ESBL-producing Escherichia coli*, *Multidrug-resistant Pseudomonas aeruginosa*, *Multidrug-resistant Acinetobacter baumannii*, *Vancomycin-resistant Enterococcus faecalis*, *Methicillin-Resistant Staphylococcus aureus*

Required Label Language**1. Care and Use of Antimicrobial Copper Containing Surface Products in Health Care Facilities**

“Product Care and Use: Antimicrobial copper containing surface products must be cleaned and disinfected according to standard practice. Health care facilities must maintain the product in accordance with infection control guidelines; users must continue to follow all current infection control practices, including those practices related to disinfection of architectural painted surfaces. This copper surface material has been shown to reduce microbial contamination but does not necessarily prevent cross contamination. This product must not be waxed, painted, lacquered, varnished, or otherwise coated by any other material.”

1. Care and Use of Antimicrobial Copper Containing Surface Products for Non-Health Care Facilities

“Product Care and Use: Routine cleaning to remove dirt and filth is necessary for standard hygiene and to assure the effective antibacterial performance of the antimicrobial copper containing surface products. Gentle cleaning agents typically used for architectural painted surfaces are permissible. The use of an antimicrobial copper surface does not replace standard good hygienic practices and/or infection control procedures. This product must not be waxed, painted, lacquered, varnished, or otherwise coated by any other material.”

Appendix A

Methodology for optional additional organisms

List of optional organisms

1. *Escherichia coli* 0157:H7 (ATCC 35150)
2. *Salmonella Enterica* (ATCC 10708)
3. *Listeria monocytogenes* (ATCC 15313)
4. *Klebsiella aerogenes* (13048)
5. *Vancomycin-Resistant Enterococcus faecalis* (ATCC 51299)
6. *Methicillin-resistant Staphylococcus aureus* (ATCC 33591)
7. *Klebsiella Pneumoniae* (ATCC 4352)
8. *Acinetobacter baumannii* (ATCC 19606)
9. *Carbapenem-Resistant Enterobacter Cloacae* (ATCC BAA-2468)
10. *ESBL (Extended Spectrum Beta Lactamase) - producing Escherichia coli* (ATCC BAA-196)
11. *Multidrug-resistant Pseudomonas aeruginosa* (ATCC BAA-2114)
12. *Multidrug-Resistant Acinetobacter baumannii* (ATCC BAA-1605)
13. *Streptococcus pyogenes* (ATCC 19615)

1) *Escherichia coli* 0157:H7 (ATCC 35150)**A. Carriers**

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up
6. Use decontaminated carriers within one week of preparation
7. All coupons are single use

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μ L of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at ~ 5000 gN for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μL of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control):** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

2) *Salmonella Enterica* (ATCC 10708)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μL of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on /unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

3) *Listeria monocytogenes* (ATCC 15313)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 48 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for $20 \pm 5 \text{ min}$ and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μL of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

4) *Klebsiella aerogenes* (13048)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $30 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μL of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $30 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $30 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $30 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $30 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $30 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

5) *Vancomycin-Resistant Enterococcus faecalis* (ATCC 51299)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers

3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth

3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min. Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 3-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99\%$ reduction (≥ 2 log reduction) down to limits of detection (complete reduction of bacteria) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

6) *Methicillin-resistant Staphylococcus aureus* (ATCC 33591)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up
6. Use decontaminated carriers within one week of preparation
7. All coupons are single use

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism

2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^{\circ}\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^{\circ}\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^{\circ}\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control):** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min. Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers

6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only

7) *Klebsiella Pneumoniae* (ATCC 4352)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μ L of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at ~ 5000 gN for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer’s ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min

4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

\log_{10} reduction achieved by the treated test carriers

(Average \log_{10} no. of organisms surviving on untreated control carriers) – (Average \log_{10} no. of organisms surviving on exposed/unexposed treated test carriers)

Neutralization Control Recovery \log_{10} difference = (\log_{10} Numbers Control) – (\log_{10} Neutralization results). Used for neutralization confirmation control only

8) *Acinetobacter baumannii* (ATCC 19606)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Plate aliquot on tryptic soy agar to grow culture. Growth observed on the surface is harvested by washing the plate with 5-10 mL of tryptic soy broth. The harvested culture is thoroughly mixed and allowed to dwell at room temperature for settling out large debris. Harvest the top portion of the liquid culture and centrifuge at ~ 5000 gN for 20 ± 5 min. Re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer’s ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min

4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log_{10} reduction achieved by the treated test carriers

(Average Log_{10} no. of organisms surviving on untreated control carriers) – (Average Log_{10} no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log_{10} difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization results). Used for neutralization confirmation control only

9) *Carbapenem-Resistant Enterobacter Cloacae* (ATCC BAA-2468)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μL of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer’s ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min

4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on exposed/unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only

10) ESBL (Extended Spectrum Beta Lactamase) - producing *Escherichia coli* (ATCC BAA-196)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μ L of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at ~ 5000 gN for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the one-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 60 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

14. Alternate incubation conditions may be needed for certain organisms. The incubation conditions may be modified to suit the test organisms if needed. If necessary, subculture plates can be stored for up to 3 days at 2-8°C prior to enumeration

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion

- F. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

G. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer’s ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The

neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure

2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

H. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 3-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99\%$ reduction (≥ 2 log reduction) down to limits of detection (complete reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers) needs to be measured in 1 hour of exposure.

I. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only

11) Multidrug-resistant *Pseudomonas aeruginosa* (ATCC BAA-2114)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 \pm 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μ L of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the one-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 60 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium

and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates

11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 3-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99\%$ reduction (≥ 2 log reduction) down to limits of detection (complete reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers) needs to be measured in 1 hour of exposure.

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on exposed/unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only

12) Multidrug-Resistant *Acinetobacter baumannii* (ATCC BAA-1605)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission
3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control

5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 μ L of the thawed stock to a tube containing 10 mL of TSB and then vortex to mix. Incubate at $36 \pm 1^\circ\text{C}$ for 18-24 hours. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use. Centrifuge at $\sim 5000 \text{ gN}$ for 20 ± 5 min and re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution

9. After all the carriers have been transferred into the neutralizer, sonicate for 5 min \pm 30 secs to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion
6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism’s ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be

prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min.
Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99.9\%$ reduction (≥ 3 log reduction) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only

13) Streptococcus pyogenes (ATCC 19615)

A. Carriers

1. Prepare 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥ 24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission

3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Uncoated scrub chart panels are used as an organism viability control
5. Place carriers in the biosafety hood for exposure to UV light for 15 ± 2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up

B. Culture preparation

Defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Plate aliquot on tryptic soy agar with 5% sheep's blood to grow culture. Growth observed on the surface is harvested by washing the plate with 5-10 mL of tryptic soy broth. The harvested culture is thoroughly mixed and allowed to dwell at room temperature for settling out large debris. Harvest the top portion of the liquid culture and centrifuge at ~ 5000 gN for 20 ± 5 min. Re-suspend the pellet in 10 mL PBS. Note: Remove the supernatant without disrupting the pellet.

C. Soil Load

Add 0.25 ml aliquot of fetal bovine serum + 0.05 ml Triton X-100 to 4.70 ml bacteria suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load. Following the addition of soil load, vortex the final test suspension for 10 seconds and immediately prior to use

D. Experimental procedure

1. Evaluate 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers
2. Coated control carriers should be evaluated concurrently with the coated test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 μ L of final test culture using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the two-hour exposure period

7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 120 ± 5 min
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of Letheen broth (neutralizer solution)- this represents the 10^0 dilution
9. After all the carriers have been transferred into the neutralizer, sonicate for $5 \text{ min} \pm 30 \text{ secs}$ to suspend any survivors from the carriers, swirl to mix.
10. Within 30 mins of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-3} for the treated carriers. Transfer the coated control carriers to neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (up to 300 colonies per plate). Incubate and enumerate with the treated carrier plates
11. Plate 1.0 mL aliquots of 10^0 dilution and 0.10 mL aliquots of the 10^0 - 10^{-3} dilutions in duplicate using standard spread plating technique on TSA plates
12. Incubate the plates at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr
13. Following incubation, count colonies and record results

E. Study Controls

1. **Purity Control:** Perform a “streak plate for isolation” on TSA plates for each final test culture and following incubation at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism
2. **Organic Soil Load Sterility Control:** Streak plate or add a sample of the soil load to a growth medium, incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and visually examine for growth. The acceptance criterion for this study control is lack of growth
3. **Carrier Sterility Control:** Add a representative un-inoculated coated test, coated control carrier to the neutralizing subculture medium. Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr and examine for growth in the subculture medium containing each carrier. The desired outcome for this study is lack of growth
4. **Neutralizer Sterility Control:** Incubate at $36 \pm 1^\circ\text{C}$ for 48 ± 4 hr an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating technique on TSA plates. The acceptance criterion for this study control is lack of growth
5. **Initial Suspension Control (Inoculum Count):** Prepare and plate serial dilutions of the culture used as inoculum. Incubate the resulting plates as in the test and then count the colonies to determine the number of organisms per milliliter of inoculum present at the start of the test. This control is for informational purposes only and therefore has no acceptance criterion

6. **Scrub chart carrier (Viability Control)** Inoculated scrub chart carriers will be used to verify the test organism's ability to survive on inert surfaces under chosen test conditions. Following the completion of the exposure time, control carriers will be transferred to neutralizing subculture media and sonicated as in the test. Ten-fold serial dilutions of the neutralizing subculture medium will be prepared, and 1.0 mL or 0.1 mL aliquots of the appropriate dilutions will be spread plated in duplicate to yield countable numbers. The plates will be incubated as in the test and enumerated. The acceptance criterion for this study control is at least 4-5 logs CFU/carrier

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. This should be conducted prior to the efficacy evaluation and reported separately. The neutralization of the coated carriers is confirmed by using treated test and untreated control carriers and the neutralizer as in the test procedure
2. Add a test carrier (one per lot) to a tube of neutralizer solution (20 mL)
3. Hold the carriers in the neutralizing solution for approximately 10 min
4. Add a 1.0 mL aliquot of a diluted suspension of the test organism yielding 10-100 CFU/0.1 mL of neutralizing subculture medium to the neutralizer, mix well. Hold for approximately 10 min. Duplicate plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates
5. A numbers control (provides baseline level of CFU for comparative purposes) is performed utilizing untreated control carriers process as indicated for the treated test carriers
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated test and numbers control should be $\leq 50\%$

G. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 unexposed, treated test carriers and 3 unexposed, untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 3-5 logs CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered effective, a $\geq 99\%$ reduction (≥ 2 log reduction) down to limits of detection (complete reduction of bacteria) in the numbers of test microbe (the difference between treated test carriers and the untreated control carriers)

H. Calculations/Data Analysis

Log reduction values are calculated based on the difference in log densities associated with the unexposed treated test carriers compared to the unexposed, untreated control carriers.

For determining the number of viable bacteria per carrier

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

For determining the geometric mean number of organisms surviving on unexposed, untreated control carriers where X equals CFU/carrier

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

For determining the geometric mean of number of organisms surviving on five unexposed treated test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3 + \text{Log}_{10}X_4 + \text{Log}_{10}X_5}{5}$$

Percent reduction achieved by the treated test carriers

$$\% \text{ reduction} = [(a-b)/a] \times 100$$

Where:

a = geometric mean of the number of organisms surviving on the untreated control carriers

b = geometric mean of the number of organisms surviving on the treated test carriers

Log₁₀ reduction achieved by the treated test carriers

(Average Log₁₀ no. of organisms surviving on untreated control carriers) – (Average Log₁₀ no. of organisms surviving on exposed/unexposed treated test carriers)

Neutralization Control Recovery Log₁₀ difference = (Log₁₀ Numbers Control) – (Log₁₀ Neutralization results). Used for neutralization confirmation control only